Faculty of Resource Science and Technology

STUDY ON LEAF ESSENTIAL OILS AND EPICUTICULAR WAXES FROM EIGHT *EUGENIA* SPECIES

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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Study on Leaf Essential Oils and Epicuticular Waxes from Eight *Eugenia* Species

ABSTRACT

Eugenia species that belong to the family of Myrtaceae were collected from various locations in Sarawak. Essential oils and epicuticular waxes were extracted from leaves of several *Eugenia* species and subsequently analyzed using capillary gas chromatography-flame ionization detector. The compound identified in the leaf essential oils and waxes in *Eugenia* species were analyzed statistically using clusters analysis to determine their correlation betwen species. The percentage of essential oils in eight species of *Eugenia* ranging from 0.1% - 2.3% (v/w) with *Eugenia christmannii* and *Eugenia* sp.A give the highest and the lowest yield, respectively. The major components identified in leaf oils of *E.* sp.A was α-curcumene (47.19%), *Eugenia* sp.B was β-caryophyllene (15.46%), *Eugenia* sp.C was methyl laurate (12.71%), while the major component for *Eugenia* sp.D and *Syzygium polyanthum* were isobornyl propionate with 6.02% and 28.75% respectively. *Syzygium aquaticum* had citronellyl isobutyrate (14.24%) as the major component. *E. christmannii* was geranyl acetone (13.71%) while *Eugenia stipulata* was dimethyl pyrazine (91.26%). The cluster analysis revealed that there was significant relationship between species *E. christmanni, E.* sp.D, *S. aquaticum* and *E.* sp.B with same chemical components existed. Leaf epicuticular waxes of *Eugenia spp.* were separated using n- hexane and the concentration of n-alkanes in the waxes was determined. The highest concentration of total n-alkanes was observed for *E.* sp.B, followed by *E.* sp.D and *E.* sp.C with 13406.85, 3216.50 and 2218.02 ng/g dry weight, respectively. No biological activity on *Artemia salina* was observed for all *Eugenia* leaf oils. However, bioassay tests on *Coptotermes* sp. have shown significant biological activities for the essential oils from *E. christmannii* and *E.* sp.D.

Keywords: *Eugenia spp.*, essential oils, n-alkanes, epicuticular waxes, gas chromatography GC/FID, clusters analysis, biological activity.

Kajian mengenai Minyak Pati dan Lilin Epikutikular dari Daun Lapan Spesies *Eugenia*

ABSTRAK

Spesies Eugenia daripada famili Myrtaceae telah dikumpul dari beberapa kawasan di Sarawak. Minyak pati dan lilin epikutikular telah diekstrak daripada daun beberapa spesies Eugenia dan seterusnya dianalisis menggunakan kromaotgrafi gas/pengesan pengionan nyalaan. Sebatian kimia dikenalpasti dalam minyak pati dan lilin epikutikular dari daun lapan spesies Eugenia telah dianlisis secara statistik menggunakan analisis gugusan bagi menentukan korelasi antara spesies. Peratusan minyak pati dalam lapan spesies Eugenia adalah dalam julat 0.1% ke 2.3% di mana hasil tertinggi dan terendah adalah dari Eugenia christmannii dan Eugenia sp.A, masing-masingnya. Komposisi kimia terbanyak dalam daun E. sp.A adalah α-curcumena (47.19%), Eugenia sp.B adalah β-kariopillena (15.46%), Eugenia sp.C adalah metil laurat (12.71%), sementara komponen terbanyak bagi Eugenia sp.D and Syzygium polyanthum adalah isobornil propionat dengan masing-masing 6.02% dan 28.75%. Syzygium aquaticum mempunyai sitronillil isobutirat (14.24%) sebagai komponen terbesar, E. christmannii adalah geranil acetone (13.71%) sementara Eugenia stipulata adalah dimethil pirazina (91.26%). Analisis gugusan menunjukkan hubungan ketara antara E. christmanni, E. sp.D, S. aquaticum dan E. sp.B dengan kehadiran komponen kimia yang sama. Lilin epikutikular dari daun Eugenia spp. dipisahkan menggunakan n-heksana dan kepekatan n-alkana dalam lilin ditentukan. Kepekatan tertinggi bagi jumlah n-alkana adalah dicerapi untuk E. sp.B diikuti dengan E. sp.D dan E. sp.C dengan kepekatan 13406.85, 3216.50 dan 2218.02 ng/g, masing-masingnya. Tiada aktiviti biologi terhadap Artemia salina untuk minyak pati dari semua spesies Eugenia. Bagaimanapun, ujian bioassei terhadap Coptotermes sp. menunjukan aktiviti biologi ketara untuk minyak parti dari E. christmannii dan E. sp.D.

Kunci kata: *Eugenia spp., minyak pati, n-alkana, lilin epikutikular, kromatografi gas GC/FID, analisis gugusan, ujian bioaktiviti.*

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

The genus of *Eugenia* that is classified in the family of Myrtacea reported to be rich in the volatile oils where they can be used for medicinal purposes (Mahmoud *et al.,* 2001). Several *Eugenia* species are not only used for medicinal purposes but they also can be used in variety of applications like spices in cooking, attractive ornamentals, edible tasty fresh fruits, dyeing purposes as well as for road side planting. Since this genus is having high value in our life, the study was carried out in determining the chemical composition of the leaf essential oils in the genus together with its leaf epicuticular waxes content. The leaves essential oils of several *Eugenia* species can be obtained through hydro distillation and subsequently analyzed by gas chromatography-mass spectrometry (GC-MS) (Cole *et al.*, 2007). GC-MS has contributed greatly to the analysis of various mixtures of organic compounds where it is certainly a useful and powerful tool within essential oil analysis (Zhao *et al.,* 2006). In many plant, essential oil samples are difficult to identify just by mass spectra alone, since two or more GC peaks have very similar or even identical mass spectra although they have different retention times and different chemical nature (Zhao *et al.*, 2006). Thus, compounds that need to identify often requires the assistance or confirmation of GC retention indices where retention time and mass spectra are complementary to each other (Zhao *et al.*, 2006).

1.2 Objectives of the project

The objectives of the study in determination of chemical composition of essential oils and epicuticular waxes in leaves of several *Eugenia spp.* are:

- ✓ To obtain essential oils from leaves part of several *Eugenia* species.
- ✓ To determine the chemical compositions of essential oils and the presence of n-alkane in leaf epicuticular waxes of several *Eugenia* species.
- ✓ To test the biological activity of essential oils from genus of *Eugenia* on termites and brine shrimp.
- ✓ To analyze statistically the relationship between the distribution of original composition in leaf oils of several *Eugenia* species.

CHAPTER TWO

LITERATURE REVIEWS

2.1 The genera of *Eugenia*

The Myrtaceae consists of about 129 genera and 4620 species (Mabberley, 1997). Myrtacea or Myrtle family is a family of dicotyledon plants which is placed within the order Myrtales, Myrtle, clove, guava, feijoa, allspices and eucalyptus. These species are woody with essential oils contained. The leaves are evergreen and the flowers have a base number of five petals while the stamens are usually very conspicuous, brightly colored and numerous. It is reported that about 3000 species are distributed in 130-150 genera where they have a distribution in tropical and warmed-temperate regions throughout the world. Historically, this family is divided into two subfamilies which are Myrtoideae and Leptospermoideae. One important member of this family is *Eugenia* which was reported as one of the larger genera with around 500 species (Mabberley, 1997). They are located throughout the world especially in the tropic and sub-tropic regions. They are natively located particularly at tropical America and Australia (Hutchinson, 1960). This genus is belonging to the kingdom plantae.

Previous of *Eugenia* has brought to light some similarities between members of this genus. They appear to be an abundance of α-pinene, β-caryophyllene and bicyclogermacrene in this genus. According to Cole *et al.* (2007), α-pinene has been reported in many *Eugenia* species. α-pinene is seen in large amount in *E. dimorpha*, *E. pluriflora*, *E. umbelliflora*, *E. uruguayensis*. β-caryophyllene and bicyclogermacrene have also appeared in many of *Eugenia* species. Although these are not reported as having the large amounts as often as α-pinene, they are still fairly abundant (Cole *et al.*, 2007). There are some compound that are common to

Eugenia in small amounts such as α-copaene, aromadendrene, α-humulene, alloaromadendrene and globulol (Apel *et al.*, 2002).

There are approximately 150 species of *Eugenia* have been identified in Malaysia. The species include *E. Aquea (jambu air), E. camtophylla, E. quadrata, E. Oleina, E. caryophyllum, E. grandis* (kelat), *E. malaccencis* and others. *E. oleina* was discovered in the jungle of Malaysia 20 years ago which is colorful and mostly used as attractive ornamental purposes. Some of *Eugenia* species can produce edible fruits that can be eaten fresh or be made into jams, jellies, juices, pies, sherbet, ice cream or wine such as *E. Aquea, E. malaccencis (jambu ball)* and *E. uniflora L.*. Several *Eugenia* species can also be used as spices and cuisine in many part of the world for instance like *E. caryophyllum, E. polyantha* and *E. aromaticum).*

2.2 Importance of *Eugenia* species

Several species of *Eugenia* are known to be rich in volatile oils which can be used as medicine. The flower buds of *E. caryophyllata* are broadly used in Chinese medicine for the treatment of many diseases such as disorders of digestive system, bacteria and fungal infections and toothache (Zhang and Chen, 1997). Several species of *Eugenia* have been reported for uses in folk medicines. *E. uniflora* which also known as pitanga fruits may be useful for preventing human diseases has been analyzed to be effective in treatment against digestive disorders and commonly use as an anti-febrile, anti-rheumatic, anti-inflammatory, diuretic and claimed to lower blood glucose levels (Kanazawa *et al.*, 2000; Ogunwande *et al.*, 2005).

The extract from pitanga leaves have been found to show pronounce anti-inflammatory action, considerable contractile activity, with a resulting effect on intestinal transit, endothelium-dependent vasorelaxant effects and hypotensive effects (Olievera *et al.*, 2006). It is also reported to inhibit the increase of plasma glucose and triglyceride levels. Some compound present in pitanga leaf extracts have also been shown to inhibit the Epstein-Barr virus, known to be closely associated with nasopharyngeal carcinoma and to have anti-microbial activity as well as anti-fungal activity (Olievera *et al.*, 2006).

Fruits from *Eugenia* species for examples like *E. uniflora* and *E. involucrate* can be eaten fresh besides of their meditational uses (Cole et al., 2007). Moreover, many of the *Eugenia* species are reported to as very rich plants in polyphenols gallic and ellargic acid derivatives (Park *et at.*, 1997; Son *et al.*, 1998), hydrolysable tannins (Tanaka *et al.,* 1996; Lee *et al.*, 1997), steroids, triterpenoid, ursane types and flavonol glycocides (Karla *et al.,* 1994) where three falvonol glycosides were isolated from *E. jambolana* roots.

Several researches conducted in India have reported that several species of this plant which are most effective and most commonly studied in relation to diabetes and their complication such as *E. jambolana*. Fruits and seeds of *E. jambolana* are trusted as an antidiabetic (Kelkar and Kaklij, 1997). Owing to these promising and diverse pharmacological effects, there is an increasing interest in the isolation and synthesis of *Eugenia* constituents.

2.3 Essential Oils

Essential oils are known as aromatic substances produced by specific plant species (Nakatsu *et al.*, 2000). Most of the oils are used as fragrance raw materials and flavoring agents. The word 'essential' is used represent the essence of the original plant. Essential oils are considered the most widely used natural products in many areas because many traditional folk medicines are based mainly on plant materials. The essential oils can be extracted from variety of trees, shrubs, herbs, grasses and flowers. Generally, essential oils contain approximately 100 components which include terpenes, alcohols, esters, aldehydes, ketones and phenols. Essential oils ring oxygen and nutrients to the tissues and assist in efficient disposal of carbon dioxide and other waste products produced by cell metabolism. They improve of the effectiveness of the immune system and decrease the blood viscosity to help with circulation. When the essential oil is consumed, it will enter and leave the body very rapidly and efficiently without leaving toxins in the body excreted through the urine and feces, perspiration and exhalation.

The chemical components contained in the essential oils and waxes can be applied for good purposes. Aromatic chemicals that are found in essential oils are derived from phenylpropane where they are linked to a cell of the body because phenylpropane is actually a precursor of amino acids which make up protein where it act as a building block for most of our body parts. Other common components of essential oils are terpinoels which are formed from acetyl coenzyme A. It plays a crucial role in the production of hormones, vitamins and energy productions.

Nowadays, it has became an important chemical material for the production of synthetic pine oils, pressure-sensitive polyterpene adhesive, insecticides, flavors and fragrance chemical (menthol, lemon and lime) and for vitamins supplement (Werger, 1984). The essential oils of *Thuya occidentalis tincture, Thymus vulgen* oil and Lavandula spica oil are used in treatments of skin diseases like acne. As well as the essential oils from the leaves and flowers like *Lippia alba*, when mixed with biological creams has been shown excellent result for treating dry skins. It is forming a barrier that regulates trans-epidermic moisture loss which is essential to the skin cell cohesion. Essential oils are commonly extracted from part of the plants by steam distillation or other processes including solvent extraction.

2.4 Epicuticular waxes

Epicuticular wax is the thin film that covers the surface of leaves in higher plants (Lemieux, 1996). The common compositions of wax contain long chain hydrocarbons, fatty acids, aldehydes, ketones, and esters (Prasad *et al.,* 1990; Jenks *et al.,* 1995). Wax is a type of lipid that is differ from fat where it lack in triglyceride esters of glycerins (propan-1,2,3-triol) and three fatty acids. The surface of wax is divided into two components, the epicuticular wax and the embedded cuticle wax. The cuticular membrane is not homogenous and it is a complex mixture of biopolymer which serves as a barrier between plant and its environment. The function of this cuticle is as the first protective barrier against UV radiation and bacterial attack (Kerstiens, 1996).

Epicuticular waxes play a role in plant biology including water loss regulation because cuticular transpiration is related to the permeability of the cuticle to water vapor and to habitat in which plants grow (Schreiber and Riederer, 1996). Shady or humid habitats frequently produce leaves more permeable to water vapor than leaves from dry or sunny exposed habitats (Bondada *et.al.*, 1996).

In addition, epicuticular waxes act as insulators from excess environmental humidity, preventing the penetration of liquid water into intercellular spaces, and avoiding the establishment of epiphylic organisms (Neinhuis and Barthlott, 1997). The composition of epicuticular waxes is dominated by long chain hydrocarbons, frequently odd-chain alkanes. Other related compounds are oxygenated derivatives such as a very long chain secondary alcohols and ketones, fatty acids, aldehydes and in many species tritepenoids appear in usually small amount (Barthlott, 1989). Each lipid class of cuticular wax may be presented as homologous series or one particular chain length may be predominated.

Cuticular wax composition varies among and within species. The same plant may show organ-to-organ differences, tissue-to-tissue differences and developmental differences. Juvenile leaves may major constituent of primary alcohol about 63%, while the mature leaves have predominantly wax ester composed of very long chain alcohols and fatty acids (42%) (Avato *et al.*, 1990). According to Gulz *et al.* (1991), they found that the composition of wax differed between young and mature leaves of *Tilia tomentosa*. The result revealed that the wax content had risen to two to three folds from young to mature leaves. During the leaves development, the synthesis of hydrocarbons, aldehydes, alcohols, fatty acids, β-amyrin and β-amyrenyl had an increasing trend. However, the synthesis of esters and acetates decreased and almost sopped after the leaves were entering to the mature phase.

Besides, the amount of epicuticular waxes may be used to identify the ecological conditions such as in drought or sun exposure, and its chemical composition may help in identification of ecologic and taxonomic groups by using clustering method. Epicuticular waxes also play an important role in restricting cuticular transpiration, of potential significance in habitats receiving large diurnal solar radiation loads (Schreiber and Riederer, 1996). In addition, waxes may protect plant from bacterial and fungal pathogen as it provide the first line of defense between the plant and its environment (Kolattukudy, 1987) and play a role in insect-interactions (Eigenbrode and Espelie, 1995). In botany, plant cuticle is covered by epicuticular wax mainly consisting of straight chain aliphatic hydrocarbons with a variety of substituted groups.

According to Gulz *et al.*, 1991, plants originated from high temperature places tend to have more waxes than those from damp or high in moisture places. Plants exposed to direct sunlight need waxy component to ensure that less water is evaporated from their leaves.

2.5 Application of essential oils and waxes for Chemotaxonomy

Chemotaxonomy is a method of biological classification based on the similarities in the structure of certain biochemical compounds among species that are being classified. The complex array and differing abundances of the chemical compounds of essential oils among the *Eugenia* species studied suggest that they might provide useful characters in understanding the phylogenic and ecological relationships among closely related species (Cole *et al.*, 2007). According to Cole *et al.* (2007), each species of *Eugenia* studied from Monterverde, Costa Rica contained different concentration of leaf essential oils. The striking differences in the mix of these compounds among the *Eugenia* species that they may provide useful characters in understanding phylogenetic relationships in this large genus whose species are notoriously difficult to classify and identify.

The study of taxonomic significance of epicuticular wax composition in genus of *Clusia* from Panama showed that group of species can be distinguished in the potential of wax composition within the genus according to the presence and diversity of terpenes and ratio of linear alkanes (Medina *et al.* 2006). These groups overlap with those generated by other classifications based on morphological and nuclear ribosomal DNA. There have been several attempts to use profile of epicuticular waxes as taxonomic criteria to differentiate groups of species within families or genera. The biosynthesis of epicuticular waxes is under close both ecological and genetic control, therefore it could provide insight into genetic differentiation among related group of species (Lemieux, 1996).

According to Madden *et al.* (1997), cluster analyses based on the percentage composition of leaf waxes resulted in the majority of populations of *Eucalyptus* species clustering according to species with excellent differentiation of many species within series. The chemical composition of leaf waxes of juvenile and adult leaves was qualitatively similar for most components and no significant quantitative differences were detected in most species. The relative ontogenetic stability of wax composition, coupled with the often clear differentiation of species, indicate the usefulness of biochemical markers derived from leaves waxes for taxonomic and phylogenetic studies in the genus *Eucalyptus*.

2.6 Bioactivity test of extracts from *Eugenia* species

Biological assay (Bioassay) is a procedure for the determination of the concentration of a particular constituent of a mixture. A Bioassay is the method of measuring the effects of biologically active substance using an intermediate in vivo or in vitro tissue or cell model under controlled conditions. Examples of biological assay are Endpoint determination and Limulus test. Endpoint determination is an establishment of the level of a quantifiable effect indicative of a biologic process. The evaluation is frequently to detect the degree of toxic or therapeutic effect.

Clove oil is an essential oil from the dried flower buds, leaves and stems of tree *Syzygium aromaticum* (Eastern Hemisphere) or *Eugenia caryophyllata* and *Eugenia aromaticum* (Western Hemisphere) (Schmid, 1972). Clove oil and its primary ingredient eugenol have been in widespread used as flavoring and fragrance agents in the U.S since before 1900. Clove oil also known as eugenol has been found to possess antibacterial, antifungal, antiviral, antitumor, antioxidant and insecticidal properties (Jirovetz *et al.*, 2006). Eugenol is slightly soluble in water and soluble in organic solvents. It is used in perfumeries, flavorings, essential oils and in medicines as a local antiseptic and anesthetic. The U.S National Toxicology Program (NTP) evaluated eugenol and found evidence of carcinogenicity in mice but not rats. The data were equivocal and not sufficient to prompt a listing of eugenol as carcinogen. There are no studies of acute or chronic adverse effects from occupational exposure to clove leaf oil or eugenol when use as a pesticide. Eugenol is not acutely toxic, with an oral LD_{50} of 2650 mg/kg in the rat. Acute and chronic clove oil toxicity to mammals is low. Acute oral LD_{50} values in animal and plant species tested were greater than 1190 mg/kg.

In subchronic toxicity test, no adverse effects were observed in studies with laboratory animals up to doses of 900 mg/kg a day. At higher dosage, liver damage has been observed but not sufficient for a listing as a carcinogen. Clove oil toxicity to insects is highly variable, with some insect orders being quite sensitive and others being quite tolerant. Eugenol is found in insect attractants as well as UV absorbers. Since it is an antioxidant it is useful in the manufacture of plastics and rubbers, besides being beneficial when ingested in a moderate amounts to keep down the free radicals. Clove oil is highly toxic to microbes, altering the cell membranes of yeast and bacteria.

2.7 Cluster Analysis

Cluster analysis is a chemometrics method, which is used to analyze the differences among species of plants (Hibert, 1997 and Otto, 1990). The term cluster analysis includes a number of different algorithms and methods for grouping objects of similar kind into respective categories. This analysis can best described as an exploratory data analysis tool which aims at sorting different objects into groups in a way that the degree of association between two objects is maximal if they belong to the same group and minimal otherwise.

Cluster analysis method has general categories that belong to it which is tree clustering, block clustering and k-Means clustering. For the study in extraction of the total amount of aliphatic hydrocarbon in leaf waxes of *Eugenia* species tree clustering method is used. Tree clustering method uses the similarities or distances between objects when forming the clusters. For the analysis in the essential oils the same clustering method is used. The compound can be classified according to their proximately in the variable space. The similarities are asset of rules that serve as criteria for grouping or separating chemical component of sample tested thus can be used for the chemotaxonomy purposes.